REMARKS

The following remarks are in response to the Examiner's Office Action mailed on April 1, 2004. Applicants express their appreciation to the Examiner for conducting telephone interviews with Applicants on July 14, 2004 and August 24, 2004. During the telephone interviews, Applicants discussed with the Examiner the issue of utility of the claimed invention under 35 U.S.C. § 101.

1. Rejection under 35 U.S.C. § 101

Claims 1-42 stand rejected under 35 U.S.C. § 101 on the ground that the claimed invention is not supported by either a specific and/or substantial asserted utility or a well-established utility. Specifically, the Examiner states that "while the claims are drawn to a method [o]f 'isolating and characterizing short-lived proteins,' the end product is not known and even when isolated and characterized, it remains to be seen just what function it serves." Applicants respectfully traverse the Examiner's grounds for utility rejection as being improper and unsupported.

Under the "Revised Interim Utility Guidelines" of the PTO and MPEP 2107 II, if at least one specific, credible, and substantial utility is provided, a rejection under 35 U.S.C. § 101 should not be made.

1) Specific Utility Provided

The claimed invention provides an efficient method for high throughput screening of a diverse protein library for short-lived proteins. As described in the Specification at page 2, lines 8-21, degradation of short-lived proteins plays important biological roles in cells, examples of which include tumor suppressor p53, oncoprotein myc, cyclins, signaling protein IκB, and key biosynthetic enzymes such as ornithine decarboxylase. To further support this asserted utility of short-lived proteins, Applicants submit herewith a review article by Ciechanover et al. (2000) (*BioEssay* 22:442-451) which summarizes many studies conducted in the relevant field of protein degradation. Ciechanover et al. describes the important roles protein degradation plays in regulation of cell cycle, modulation of the immune and inflammatory responses, control of

signal transduction pathways, development and differentiation. *See* Abstract. In particular, Ciechanover et al. elucidates that many short-lived proteins involved in human diseases are degraded via ubiquitin-mediated proteolysis, such as the tumor suppressor protein p53, cyclin-dependent kinase (CDK) inhibitor p27Kip1, cystic fibrosis (CF) gene encoding the CFTR (CF transmembrane conductance regulator), etc. *See* page 448, under the section entitled "The ubiquitin system and pathogenesis of human diseases." Furthermore, as stated in Dr. Li's Rule 1.132 Declaration, the claimed method has been used successfully to screen cDNA libraries for genes encoding short-lived proteins of biological and therapeutic significance. Examples of such short-lived proteins include cervical cancer 1 proto-oncogene protein, insulin-like growth factors, serine proteinase inhibitor, etc. Thus, the claimed invention has at least one specific utility for efficiently screening protein libraries for short-lived proteins which have a variety of applications, such as serving as targets for disease diagnosis and therapeutic intervention, as asserted in the Specification at page 11, lines 1-13.

2) Substantial Utility Provided

A "substantial utility" is defined by the PTO Training Materials for the "Revised Interim Utility Guidelines" and MPEP 2107.01 I as a "real world" use. An assay method for identifying short-lived proteins that themselves have a "substantial utility" is considered to be a "real world" use.

As discussed above, the claimed method provides a high throughput assay for screening short-lived proteins from a library of diverse proteins. The screening assay is a very useful tool for artisans in biotech research and industry to select and isolate short-lived proteins of great importance in therapeutics and diagnosis of diseases. As explained in Dr. Li's Declaration, the assay is robust and high throughput because it can be performed without using ubiquitin conjugation as a search criterion. This feature of the invention allows for expanded search for important proteins that are labile but does not require ubiquitin modification for their turnover in the cell.

The resulting selected proteins can be used in diagnostic applications and for treating specific diseases in the clinic. Applicants therefore submit that a "real world" use demonstrating a substantial utility has also been provided.

3) Credible Utility Provided

The claimed method also has credible utility. It is well known that short-lived proteins such as tumor suppressor p53 and proto-oncogenes are extremely useful for the diagnosis and treatment of diseases such as cancer. The claimed high throughput assay for screening short-lived proteins would be readily recognized by skilled artisans in the relevant field to have credible utility as a useful tool for searching protein targets for diagnostic and therapeutic purposes. Applicants therefore submit that a credible utility has also been provided.

In view of the specific, credible, and substantial utility of the claimed method, the pending utility rejection should be withdrawn.

2. Rejections under 35 U.S.C. § 112, First Paragraph

1) Written Description

Claims 1-42 are also rejected under 35 U.S.C. § 112, First Paragraph for insufficient written description. Specifically, the Examiner states that "[w]hile the specification does provide drawings, and make reference t[o] them in the prophetic examples, the figures lack the full, clear, concise and exact terminology so as to clearly identify the starting materials and methods of operation that permit the full scope of the invention to be practiced." Applicants respectfully traverse the Examiner's grounds for rejection based on the following reasons.

The instant application provides adequate written description of the claimed invention in the text and drawing parts of the Specification. As described on pages 12-14, a general scheme of the screening assay for short-lived proteins is provided and illustrated in Figure 1. As illustrated in Figure 1, all of the steps in the claimed method are described including expression of the diverse fusion protein library, modification of the rate of protein expression, and selection of cells based on reporter signal intensities. In the following subsections (starting at page 16),

the Specification provides detailed description of each of the claim elements, including 1) formation of reporter-cDNA fusion protein construct library; 2) formation of vector library comprising reporter-cDNA fusion protein constructs; 3) formation of library of cells comprising reporter-cDNA fusion protein constructs; 4) sorting cell library into population based on reporter signal intensity; and 5) selecting cells by inhibiting protein expression and/or protein degradation. As further supported by Dr. Li's Declaration, by following the screening assays described in the Specification, short-lived proteins from human cDNA libraries were selected and characterized.

For the reasons set forth above, Applicants submit that the claimed invention is supported by adequate written description under 35 U.S.C. § 112, First Paragraph. Withdrawal of this ground of rejection is therefore respectfully requested.

2) Enablement

Claims 1-42 are also rejected under 35 U.S.C. § 112, First Paragraph for lack of enablement. Specifically, the Examiner states that "[c]laims 1-42 are not enabled by the disclosure and to do so, which would require the identification and development of starting materials and reaction conditions would cause the skilled artisan to resort to trial-and-error experimentation." Applicants respectfully traverse the Examiner's grounds for rejection based on the following reasons.

The claimed invention is a screening assay for short-lived proteins which comprises the following steps:

> i) expressing a fusion protein in each cell within a library of cells, the fusion protein comprising a reporter protein and a protein encoded by a sequence from a cDNA library derived from a sample of cells, and the sequence from the cDNA library varying within the cell library;
>
> ii) inhibiting further expression of the fusion protein to allow the expressed fusion protein to degrade in the cell; and
>
> iii) selecting a population of cells from the library of cells based on the population of cells having different reporter signal intensities than other cells in the library, the difference being indicative of the population of cells expressing

shorter lived fusion proteins than the fusion proteins expressed by the other cells in the library.

The specification provides ample teaching of how to construct a library of cells expressing diverse fusion proteins (pages 16-20; e.g., by expressing in COS-7 cells a library of pd2EGFP vectors carrying a human cDNA library, page 19, lines 30-32); how to inhibit expression of the fusion proteins (pages 21-22; e.g., by using cycloheximide, p. 21, line 27); and how to select a population of cells based on different reporter signal intensities (pages 21-23; e.g., selecting the cells based on 50% reduction in reporter signal intensities, page 23, lines 2-3). As further supported by Dr. Li's Declaration, by following the screening assays described in the Specification, short-lived proteins from human cDNA libraries were selected and characterized.

For the reasons set forth above, Applicants submit that the claimed invention is enabled under 35 U.S.C. § 112, First Paragraph. Withdrawal of this ground of rejection is therefore respectfully requested.

CONCLUSION

In light of the amendments and remarks set forth above, Applicants earnestly believe that they are entitled to a letters patent, and respectfully solicit the Examiner to expedite prosecution of this patent application to issuance. Should the Examiner have any questions, the Examiner is encouraged to telephone the undersigned.

Respectfully submitted,

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Ubiquitin-mediated proteolysis: biological regulation via destruction

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Summary

The ubiquitin proteolytic system plays an important role in a broad array of basic cellular processes. Among these are regulation of cell cycle, modulation of the immune and inflammatory responses, control of signal transduction pathways, development and differentiation. These complex processes are controlled via specific degradation of a single or a subset of proteins. Degradation of a protein by the ubiquitin system involves two successive steps, conjugation of multiple moieties of ubiquitin and degradation of the tagged protein by the 26S proteasome. An important question concerns the identity of the mechanisms that underlie the high degree of specificity of the system. Substrate recognition is governed by a large family ubiquitin ligases that recognize the substrates, bind them and catalyze/facilitate their interaction with ubiquitin. ***BioEssays*** **22:442–451, 2000. © 2000 John Wiley & Sons, Inc.**

Introduction

That modification by Ubiquitin provides a proteolytic signal was discovered during biochemical fractionation-reconstitution studies of rabbit reticulocyte lysates while studying ATP-dependent selective degradation of abnormal/misfolded proteins (reviewed in Ref. 1). Numerous molecular, biochemical, cellular, genetic and clinical studies have since unraveled the major role that ubiquitin-mediated proteolysis plays in a broad array of basic cellular processes. Among these are regulation of the cell cycle, differentiation and development, the cellular response to extracellular effectors and stress, modulation of cell surface receptors and ion channels, DNA repair, regulation of the immune and inflammatory responses and biogenesis of organelles. Considering these numerous processes, it is not surprising that the system has been implicated in the pathogenesis of many diseases. In most cases, modification by ubiquitin targets the substrate for degradation by the 26S proteasome but, in certain cases, modification leads to targeting to the lysosome/vacuole. In contrast, the more recently discovered modification by ubiquitin-like proteins serves non-proteolytic functions such as routing of cellular proteins to their subcellular compartments. The list of cellular proteins targeted by ubiquitin is growing rapidly. Among them are cell cycle regulators, tumor suppressors and growth modulators, transcriptional activators and their inhibitors, cell surface receptors and endoplasmic reticulum proteins. Mutant proteins or otherwise damaged proteins are recognized specifically and, unlike their normal counterparts, removed rapidly.

Degradation of a protein via the ubiquitin-proteasome pathway involves two successive steps: (1) covalent attachment of multiple ubiquitin molecules to the substrate; and (2) degradation of the tagged protein by the 26S proteasome and recycling of ubiquitin via the activity of ubiquitin C-terminal hydrolases (isopeptidases; see Fig. 1 for a scheme of the ubiquitin pathway). For recent reviews, see Refs 2–10. Conjugation of ubiquitin proceeds via a three step mechanism. Initially, the ubiquitin-activating enzyme, E1, activates the C-terminal Gly of ubiquitin to a high energy thiol ester with an internal E1 Cys residue. One of several E2 enzymes (ubiquitin-carrier proteins or ubiquitin-conjugating enzymes, Ubc's) transfers the activated ubiquitin, via an E2 ubiquitin thiol ester intermediate, to the substrate that is specifically bound to a member of the ubiquitin-protein ligase family, E3. Transfer can be either directly to the substrate, or via an additional E3 ubiquitin thiol ester intermediate. E3s facilitates/catalyzes covalent attachment of ubiquitin to the substrate. The first moiety is transferred to an ε-NH_2 group of internal Lys residue or to the α-NH_2 group[11] of the substrate to generate an isopeptide or a linear peptide bond,

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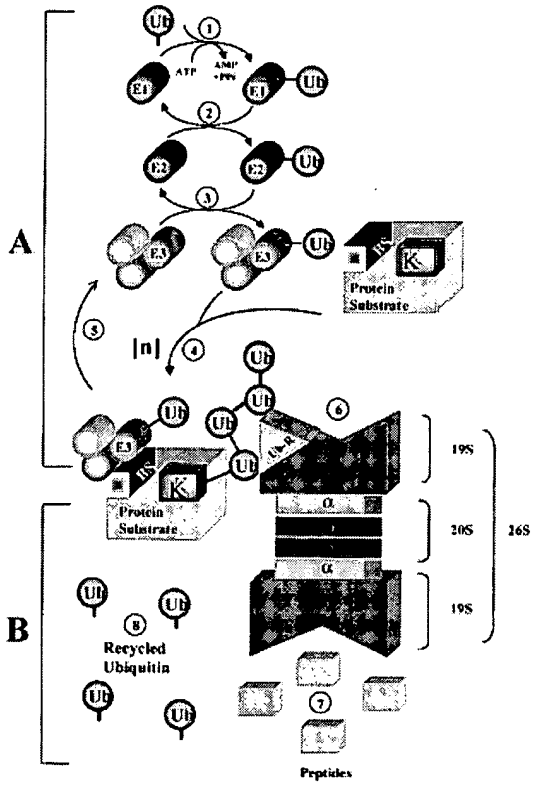

Figure 1. The ubiquitin-proteasome pathway. **A:** Conjugation of ubiquitin to the target substrate. (1) Activation of ubiquitin by the ubiquitin-activating enzyme, E1. (2) Transfer of the activated ubiquitin moiety to a member of the ubiquitin-carrier protein (ubiquitin-conjugating enzymes; Ubc's) family of enzymes, E2. (3) Transfer of activated ubiquitin from E2 to a Cys residue on E3 (in the case of the HECT domain family of ligases) or directly to the substrate (in most other cases; not shown). (4) Complex formation between the substrate and a specific binding site (BS) on the ubiquitin ligase, E3, followed by transfer of the first ubiquitin moiety to an internal Lys residue (K) of the substrate and subsequent formation of a polyubiquitin chain. E3 can be either a monomer, a homodimer, or a component of a larger multimeric complex (as depicted in the Figure). **B:** Degradation of the polyubiquitinated substrate by the 26S proteasome complex. (6) The polyubiquitinated substrate binds to the ubiquitin receptor (Ub-R) subunit of the 19S complex and is then degraded to short peptides (7) with the release of free and reutilizable ubiquitin (8). Free E3 is also recycled (5). The 26S proteasome is composed of two 19S regulatory complexes attached at each side to the barrel-shaped 20S catalytic complex.

respectively. In successive reactions, a polyubiquitin chain is synthesized by transfer of additional activated ubiquitin moieties to an internal Lys residue of the previously conjugated ubiquitin molecule. While commonly, Lys_{48} of ubiquitin is utilized for cross-linking, linkages to Lys_{63}[12] or Lys_{29}[13] have also been described, though they may have distinct functions. The chain serves, most probably, as a recognition marker for the protease.

The structure of the ubiquitin system is hierarchical: a single E1 species activates all the ubiquitin required for all modifications and transfers ubiquitin to several isoforms of E2. Each E2 isoform is able to act with either one or several E3 proteins. A limited number of E3 enzymes have been described thus far, but it appears that this is a large and rapidly growing family of proteins. E4 has been described recently and is involved in polyubiquitin chain elongation. Its activity, however, appears to be restricted to a limited subset of substrates.[14]

Although the components of the ubiquitin system have been localized to the cytosol and nucleus, targets of the system are known to include membrane-anchored and even ER lumenal proteins. These proteins are 'retro-transported' to the cytosol, ubiquitinated, and degraded by the proteasome (reviewed in Refs. 15,16).

An important problem involves the mechanisms that underlie the high specificity of the system. Why are some proteins extremely stable while others are short-lived? And, why are some proteins degraded at a particular time during the cell cycle, or only following specific extracellular stimuli, while they are stable under most other conditions? Specificity is imparted by two distinct groups of proteins. Within the ubiquitin system, protein substrates must be recognized and bind to an E3 enzyme prior to their modification. Recognition is mediated by specific structural motifs within the substrate. Some of these motifs are encoded within the protein itself and the proteins that harbor them are degraded constitutively. Stability of other proteins depends on their state of oligomerization, post-translational modification, such as phosphorylation, or association with ancillary proteins such as molecular chaperones, that act as recognition elements in *trans*. Some transcription factors must dissociate from the specific DNA to which they bind in order to be recognized. In other cases, it is the E3 enzyme or a subunit of the E3 complex that must be modified in order to be active (see Fig. 2 for the different modes of recognition). Thus, in addition to the central role of E3 proteins in the recognition process, the modifying enzymes, ancillary proteins, and specific DNA binding sites also play an important role. It is rare that a single protein is targeted by a specific E3 ligase, and in most cases, an E3 recognizes a subset of proteins that contain similar structural motifs. Some proteins are recognized by two different E3 enzymes, via distinct recognition motifs.

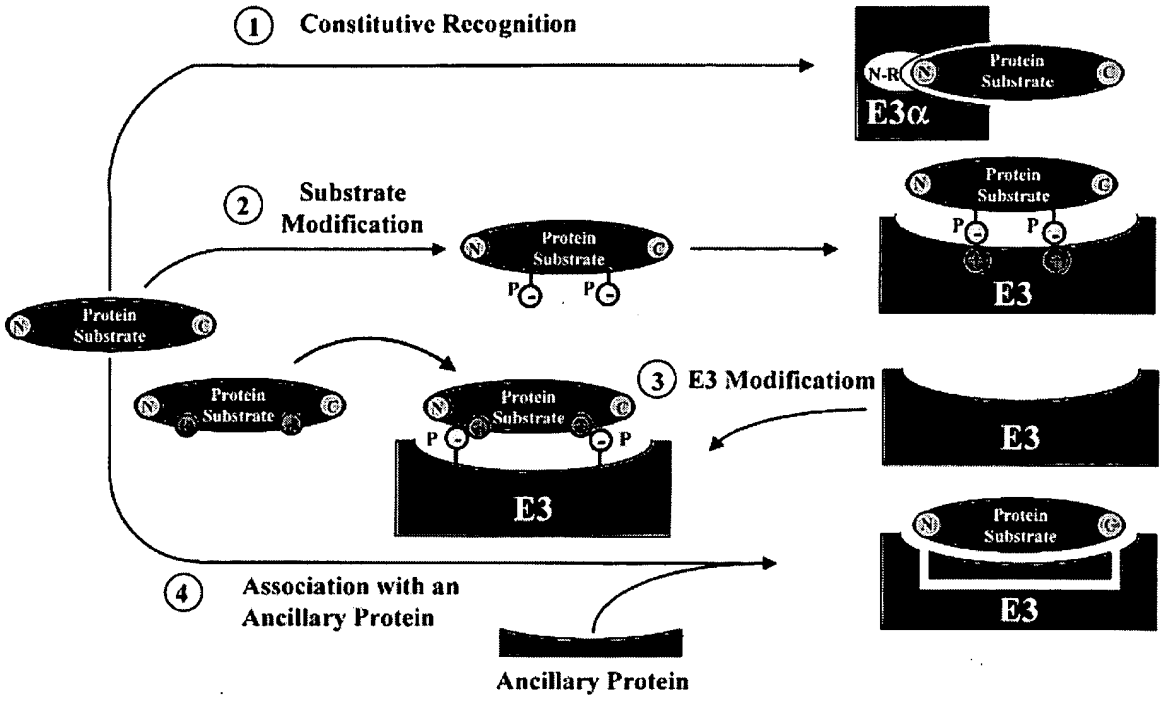

Figure 2. Modes of recognition of protein substrates by the different E3s. **1:** Constitutive recognition via a primary motif (for example, E3α/Ubr1 constitutively targets proteins following recognition of their N-terminal residue or an internal primary motif). **2:** Recognition of a substrate following its post-translational modification (for example, IκBα or β-catenin are targeted by the $SCF^{\beta\text{-}TrCP}$ ligase complex following phosphorylation). **3:** Recognition of a substrate following post-translational modification of the E3 (e.g. mitotic cyclins are targeted by the APC/cyclosome, following phosphorylation of an unidentified component within the ligase complex). **4:** Recognition of the proteolytic substrate following its association with an ancillary protein (e.g. p53 is targeted by E6-AP following association with HPV-E6 types 16 or 18, or Hsc70 that can target certain misfolded proteins).

Enzymes of the ubiquitin system

E1 and E2s

A single E1 catalyzes all ubiquitination reactions, and inactivation of this gene is lethal. The yeast genome encodes 11 E2 enzymes denoted Ubc1–8, 10, 11, 13; Ubc9 and Ubc12, although members of the Ubc family, are involved in conjugation of ubiquitin-like proteins. E1 and E2 can catalyze transfer of a single ubiquitin moiety to target proteins in vitro. The role of mono-ubiquitin adducts is not known as they are not recognized by the 26S proteasome and catalysis of polyubiquitination that renders the substrate susceptible for degradation requires E3.

Ubiquitin ligases, E3s

It is the E3 ligases that provide the high specificity of the system, although these are the least defined components of the pathway. The ligase is a protein or a protein complex that binds both the E2 and the substrate. Interaction with the substrate is either direct or via an ancillary protein. Most often, the E3 serves as a scaffold protein that brings together the E2 and the substrate. In some cases, the activated ubiquitin is transferred from E2 to an internal Cys residue on E3 prior to its conjugation to the target. Here, the E3 has a catalytic role. E3 ligases can be subdivided into at least six subtypes with regard to structure and/or class of signals they recognize.

(1) E3α (Ubr1 in yeast; reviewed in Ref. 17) recognizes 'destabilizing' N-terminal residues ('N-end rule' substrates). The physiological significance of the 'N-end rule' pathway is not clear, since it is not essential in yeast, and only a few natural substrates traverse it. E3α has two defined binding sites, for basic and bulky hydrophobic N-terminal residues. Importantly, it also recognizes proteins via internal, downstream signals. E3β is a ligase related to E3α that targets proteins with small uncharged N-terminal residues.[18]

(2) HECT-domain proteins (homologous to E6-AP carboxyl terminus) contain a 350 residue domain homologous to the C-terminal domain of the prototypical member of the family, E6-AP (E6-associate protein).[19] This domain contains a

conserved Cys residue to which the activated ubiquitin moiety is transferred from E2.[20] E6-AP targets p53 for rapid degradation in the presence of the human papillomavirus (HPV) oncoprotein E6.[21] It also targets native cellular proteins, such as Blk (a member of the Src family of kinases) in the absence of E6.[22] Other HECT-domain E3 ligases target the kidney epithelial Na^+ channel,[23] the yeast uracil and amino acid permeases,[24] and SMADs specific to the bone morphogenetic protein (BMP) pathway.[25]

(3) The anaphase promoting complex (APC)[26] or cyclosome[27] consists of at least eight subunits[28] and targets mitotic substrates. While several subunits of the APC contain tetratricopeptide (TPR) repeats that are involved in protein-protein interactions, the identity of the E3 subunit has remained elusive. At least two, sub-stoichiometric, ancillary factors have been identified that regulate its activity. The yeast proteins are Cdc20 and Hct1/Cdh1,[29] whereas the human homologs are p55Cdc/hCdc20 and hCdh1.[30] These proteins confer substrate specificity to APC. For example, Cdc20-APC is active at the beginning of anaphase, when it degrades the anaphase entry inhibitor Pds1, whereas Hct1/Cdh1 is required for the degradation of cyclin B at the end of mitosis. Both proteins are regulated. Failure of spindle assembly leads to binding of the Mad1-3 checkpoint protein to Cdc20, which results in inhibition of mitosis.[31] CDK/Cyclin B phosphorylates Hct1/Cdh1 which inhibits its binding to APC.[32] APC itself is a target of an activating phosphorylation by cyclin B/Cdk1.[33] The best-characterized APC substrates are the A- and B-type cyclins. These, and other, APC substrates contain a degradation signal designated the 'destruction box' which has the following consensus sequence, **R**-A/T-A-**L**-G-X-I/V-G/T-N (indispensable residues are in bold; reviewed in Ref. 8). The role of this sequence is, however, still obscure.

(4) The SCF complexes (Skp1, yeast Cdc53, or mammalian Cullin, and F-box protein) act with the E2 Cdc34/Ubc3,[9] and possibly with members of the UbcH5.[34] Recently, a fourth, Rbx1,[35] or Roc1,[36] and also, potentially a fifth, Sgt1[37] component of this complex have been identified. The catalytic complex may have the following hexameric structure: E2 · Rbx1/Roc1 · Cdc53/Cullin-1 · Skp1 · F-Box protein · Protein substrate. The role of Sgt1 is not yet clear. The Rbx1/Roc1, Skp1, and Cdc53/Cullin-1 subunits are probably common to all SCF complexes. The F-box protein which binds specific substrates is the variable component, and the different complexes are designated according to the F-box component (e.g. SCF^{Cdc4}, $SCF^{\beta\text{-}TrCP}$, SCF^{Skp2}). $SCF^{\beta\text{-}TrCP}$ targets phosphorylated IκBα,[38] β-catenin, [39] and HIV-1-Vpu[40] (binding of Vpu forms a ternary complex, Vpu-CD4-SCF, with subsequent degradation of the CD4 receptor). The signal recognized by $SCF^{\beta\text{-}TrCP}$ in all three substrates is DS(P)GΨXS(P), though other potential substrates, which are yet to be identified, could contain a different signals. SCF^{Skp2} targets E2F-1[41] and $p27^{Kip1}$.[42] Although $p27^{Kip1}$ must be phosphorylated on Thr_{187} in order to be recognized, it is not clear whether targeting of E2F-1 also requires phosphorylation.

(5) Several ring finger proteins also appear to serve as ubiquitin ligases.[43] For example, the c-Cbl proto-oncoprotein stimulates CSF-1 receptor ubiquitination and endocytosis.[44] Similarly, it targets the EGF and PDGF receptors.[45,46] The ring finger domain plays an important role in the function of the ligase, possibly in the formation of the polyubiquitin chain.[47]

(6) The von Hippel-Lindau tumor suppressor protein (pVHL) is part of a complex that includes Elongins B and C, Cullin-2, and Rbx1/Roc1 and is similar to SCF complexes. This complex can catalyze polyubiquitination,[48,49] and the recent discovery that it targets hypoxia-inducible factors (HIFs) for degradation[50] raises the possibility that it serves as their ligase.

The 26S proteasome

The proteasome is a large, 26S, multicatalytic protease that degrades polyubiquitinated proteins to small peptides (reviewed recently in Refs. 5,6). It is composed of two subcomplexes, a core catalytic 20S particle and a regulatory 19S particle. The 20S complex is barrel-shaped and consists of four stacked rings, two identical outer α rings and two identical inner β rings. The α and β rings are composed each of seven distinct subunits, giving the complex the general structure of $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. The catalytic sites are localized in some of the β subunits. Electron microscopy analysis shows that each extremity of the 20S complex is capped by a 19S particle. The yeast 19S complex has been further resolved into two sub-complexes, the 'base', which consists of 6 ATPases of the AAA family and three additional proteins, and an 8-subunit 'lid' that is homologous to copsignalosome complexes.[51] The function of the signalosomes is not known, and, therefore, the significance of the homology is also an enigma.

One function of the 19S complex is to recognize ubiquitinated proteins and other substrates of the proteasome. A ubiquitin-binding subunit, Rpn10/Mcb1, has been identified in the 19S particle. Since this subunit is not essential in yeast, however, [52] there must be additional ubiquitin-binding component(s) in the 19S complex. A second function of the 19S complex, probably conferred by the ATPase subunits, is to create a 'gate' in the α ring through which substrates can be inserted into the proteolytic chamber: the yeast 20S particle seems to be occluded at both ends by the protruding termini of the α subunits.[53] Finally, it is assumed that the 19S particle unfolds the polypeptide chain so it can be inserted into the proteolytic chamber. Interestingly, the 'base' sub-complex exhibits a chaperone-like activity in refolding denatured proteins.[54] Thus, during proteolysis it may act in an 'opposite' direction.

Deubiquitinating enzymes

The cell contains many deubiquitinating enzymes that cleave ubiquitin molecules conjugated via either isopeptide or linear peptide bonds. These processes are essential for the maturation of newly synthesized ubiquitin molecules which are often translated as a linear, 'head' to 'tail', polyubiquitin molecules, for the release of some ribosomal proteins that are synthesized fused at their N-terminal residue to a ubiquitin moiety that targets them to the ribosome, and for the recycling of ubiquitin molecules after degradation of the substrate (reviewed in Ref. 55). Inactivation of the major deubiquitinating enzymes results in inhibition of ubiquitin-mediated proteolysis. This is probably a consequence of two effects: depletion of the cellular free ubiquitin pool, and saturation of the proteasome with cleaved, substrate-free, polyubiquitin chains. Deubiquitinating enzymes can also accelerate proteolysis by 'trimming' polyubiquitin chains to a length that is most efficiently recognized by the 26S proteasome. Deubiquitination may also serve a 'correction' function by releasing ubiquitin chains from substrates that have been spuriously conjugated.

Deubiquitination also has specific regulatory roles. For example, specific deubiquitinating enzymes affect transcriptional silencing in *S. cerevisiae*[56] and eye development in *Drosophila*.[57] DUB(deubiquitinating)-1 and DUB-2 are induced by cytokines, and high-level expression of DUB-1 leads to cell-cycle arrest.[58] An intragenic deletion in the gene encoding the ubiquitin-carboxyl-terminal hydrolsae (UCH) isozyme Uch-L1 results in the gad (gracile axonal dystrophy) syndrome in mice.[59] This is characterized by sensory and motor ataxia and neurodegeneration accompanied by the accumulation of ubiquitin conjugates (see also below for mutations in human UCH-L1). In all these cases however, the target proteins remain, as yet, unknown.

Sites of intracellular degradation

Ubiquitin-mediated degradation of cytosolic and membrane proteins occurs in the cytosol and on the cytosolic face of the ER membranes (reviewed in Refs. 15,16). Although components of the system have been localized to the nucleus, conjugation and degradation have not been demonstrated in this organelle. Leptomycin B, a drug that inhibits nuclear export, almost completely prevents Mdm2- and E6-AP/E6-dependent degradation of p53.[60] The degradation of $p27^{Kip1}$ probably occurs also in the cytosol, following Jab1 (p38)-mediated nuclear export.[61] The physiological significance of regulation via nuclear-cytoplasmic shuttling and of the physiological role of the components of the ubiquitin pathway in the nucleus are still mysterious.

'Alternative' pathways

As described above, the two 'arms' of the ubiquitin-proteasome pathway are polyubiquitination of the substrate followed by its proteasome-mediated degradation. The two arms of the pathway can also function independently, however. For example, ornithine decarboxylase (ODC) is degraded by the proteasome without prior ubiquitination. It appears that antizyme, an inhibitor of ODC, mediates recognition of non-ubiquitinated ODC by the proteasome.[62] In other cases, ubiquitination of membrane receptors or transporters results in their internalization and degradation in the vacuole/lysosome (reviewed in Refs. 15,16,63). Unlike proteasomal degradation, which requires polyubiquitination, mono-ubiquitination appears to be sufficient for receptor internalization, at least in some cases. Furthermore, mono-ubiquitination can occur on Lys residues other then Lys_{48}, for example, the mono-ubiquitination required for endocytosis of certain membrane receptors can occur on Lys_{63} (reviewed in Ref. 63). One exception to the rule that ubiquitination leads to protein degradation is the limited processing of p105, the precursor of the transcription factor NF-κB. The C-terminal domain of the molecule is degraded specifically, leaving behind p50, the active subunit of the factor. Cleavage requires the appropriate positioning of three domains, a Gly-rich region that acts as a processing 'stop' signal,[64] a ubiquitination/E3 anchoring domain,[65] and a C-terminal domain that is regulated by phosphorylation.[66]

Regulation of ubiquitin-mediated proteolysis

General regulation

The ubiquitin pathway can potentially be regulated at the level of either ubiquitination or proteasome activity. It appears that the general components of the system, E1, E2s, and the proteasome, are constitutively active, or may be regulated to only a limited extent. This is, perhaps, to be expected, since these components are involved in the degradation of a multitude of substrates, and alteration in their activity will affect many substrates. Regulation of the general components have, however, been demonstrated in two cases. One is the up-regulation of the ubiquitin pathway to achieve bulk degradation of skeletal muscle proteins that occurs in different pathophysiological conditions such as fasting, cancer cachexia, severe sepsis, metabolic acidosis, or following denervation (reviewed in Ref. 67; see also below).[67] This occurs also during specific developmental processes, such as insect metamorphosis where massive breakdown of larval muscle tissue occurs prior to the development of the pupa and the mature butterfly.[68] The second example of a change in the general components of the system occurs following treatment with IFN-γ. This cytokine induces changes in the subunit composition of the 20S proteasomal complex. Consequently, the antigenic peptides that are generated following proteosomal degradation have higher affinity for the presenting MHC class I molecules and for the cytotoxic T-cell receptor (reviewed in Ref. 7).

Specific regulation

Degradation of specific substrates is regulated mostly at the level of ubiquitination. Regulation can be mediated via post-translational modification or a structural change of the substrate that render it susceptible to recognition by the E3 ligase, or via modulation of the activity of specific E2/E3 complexes. From studies thus far, it appears that the mode of regulation correlates with the class of E3. Ubiquitination by SCF complexes requires phosphorylation of the substrate, the APC activity is modulated by the presence of activators and by phosphorylation, and the activity of at least some HECT-domain proteins towards certain substrates may depend on ancillary proteins that facilitate recognition in *trans*.

Regulation by modification of the substrate: Phosphorylation of many substrates is required for their recognition by their E3s. Conversley, similar modification of many other proteins prevents this. Substrates that require prior phosphorylation include the yeast G1 cyclins, Cln2 and Cln3, the yeast cyclin-dependent kinase (CDK) inhibitors, Sic1 and Far1, the mammalian G1 regulators, cyclins D and E, the mammalian CDK inhibitor, $p27^{Kip1}$, the mammalian transcriptional regulators, IκBκ and β-catenin, and the viral protein HIV-1-Vpu. In all of these cases, the E3 was identified as a member of the SCF family of complexes (reviewed in Refs. 2–4,9). Proteolysis of IκBα, β-catenin and binding to HIV-1 Vpu is mediated by $SCF^{β\text{-}TrCP}$ following specific phosphorylation of two serine residues that reside within a defined consensus sequence DS(P)GΨXS(P).[38–40] The activity of the ligase towards the substrates is constitutive, and the regulatory step is the phosphorylation (in the case of IκBα) or dephosphorylation (in the case of β-catenin). Unlike β-TrCP, no consensus recognition motif(s) have been defined for other F-box proteins. Sic1 and $p27^{Kip1}$ are G1 CDK inhibitors, which must be degraded in order to allow entry into the S phase. Phosphorylation at several Thr and Ser residues in the N-terminal domain of Sic1 and phosphorylation of $p27^{Kip1}$ at Thr_{187} are required for their recognition by SCF^{Cdc4} and SCF^{Skp2}, respectively. Here too, the SCF complexes are constitutive, and phosphorylation of the substrates is the regulated step.

Interestingly, ubiquitination of an SCF substrate can also be modulated by the presence or absence of the specific F-box protein. E2F-1 and $p27^{Kip1}$ are ubiquitinated by SCF^{Skp2}.[41,42] Skp2 expression and activity are regulated during the cell-cycle, with a peak of expression during S phase.[69] Modulation of the expression of the F-box protein might confer an added level of regulation.

Degradation of the proto-oncogene c-mos by the ubiquitin pathway is inhibited by phosphorylation on $Ser^{3(70)}$. Interestingly, activation of c-mos leads to phosphorylation and stabilization of c-fos, another substrate of the ubiquitin pathway.[71] Another example is that of the anti-apoptotic protein Bcl-2. Dephosphorylation of Bcl-2 following apoptotic stimuli renders it susceptible to degradation by the ubiquitin pathway.[72]

Regulation by modulation of ubiquitination activity: Regulated degradation of specific classes of substrates could be achieved by modulation of the activity of the ubiquitination machinery. For example, it has been shown recently that degradation of mitotic regulators by the APC is regulated by different activators and inhibitors and by phosphorylation (Refs. 29–33 and see above).

Regulation by ancillary proteins: Several viral proteins exploit the ubiquitin system by targeting for degradation cellular substrates which may interfere with propagation of the virus. In some instances, the viral protein functions as a 'bridging' element between the E3 and the substrate, thus conferring recognition in *trans*. The prototype of such a protein is the high risk HPV oncoprotein E6 which interacts with an E6-AP HECT domain E3, and with the tumor suppressor protein p53. This interaction targets p53 for rapid degradation and, thus, most probably prevents stress signal-induced apoptosis and ensures further replication propagation of the virus (Ref. 21 and see above). In a different case, the Vpu protein of the HIV-1 virus is recognized by the F-box protein, β-TrCP.[40] Vpu also binds to the CD4 receptor in the ER of T cells infected by the virus. This leads to ubiquitination and subsequent degradation of CD4 by the $SCF^{β\text{-}TrCP}$ complex, thus enabling the virus to escape from immune surveillance.

Degradation of many misfolded/denatured proteins is mediated by formation of a complex with molecular chaperones such as Ydj1 in yeast[73] and Hsc70 in mammals.[74] If the chaperone is unable to refold a denatured substrate, despite several repeated cycles of association and dissociation, it presents the misfolded protein to the ligase for ubiquitination and subsequent degradation. This is the 'refold or degrade' function of the chaperone.

Regulation by masking of a degradation signal: The presence of either one of two transcription factors, MATa1 and MATα2, determines the mating type of haploid yeast cells. The diploid cell expresses both a1 and α2 that form a heterodimer with distinct DNA-binding specificity. In haploid cells, the two factors are rapidly degraded by the ubiquitin system. Degradation of α2 requires two degradation signals, Deg1 and Deg2. Strikingly, both a1 and α2 are stabilized by heterodimerization.[75] For α2 at least, it has been shown that residues required for interaction with a1 overlap with the Deg1 degradation signal and it is possible that binding of a1 interferes with the degradation of α2 by masking the ubiquitin recognition signal. A similar mechanism is thought to protect the *Drosophila* homeobox protein Homothorax in cells expressing its binding partner Extradenticle.[76] An analogous observation involves the transcription factor MyoD which is protected from conjugation by binding to its cognate DNA sequence.[77]

The ubiquitin system and pathogenesis of human diseases

As mentioned above, the ubiquitin system has been implicated, both directly and indirectly, in the pathogenesis of several important human diseases. Angelman syndrome is characterized by severe motor and mental retardation and is caused by mutations in the ubiquitin ligase E6-AP.[78] The substrate(s) that accumulates and is toxic to the developing brain cells has not been identified. Liddle's syndrome, is a severe form of hypertension that is due to a mutation in the PPxY motif of the kidney epithelial Na^+ channel (ENaC). The mutant channel is unable to interact with the WW domain of its ubiquitin ligase Nedd4 and the consequent stabilization of the channel leads to increased reabsorption of Na^+ and H_2O (Ref. 23 and see above). Targeting of the tumor suppressor protein p53 for degradation by the human papillomavirus oncoprotein E6 and the ubiquitin ligase E6-AP has been implicated in the pathogenesis of human uterine cervical carcinoma.[21,79]

Less direct evidence implicates the ubiquitin system in the pathogenesis of many other diseases. The level of the cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$, which acts as a negative growth regulator/tumor suppressor, is inversely correlated with the aggressiveness and severity of many types of malignancies, including colorectal, breast, and prostate carcinomas (reviewed in Ref. 80). Interestingly, the low level of p27 in the most aggressive tumors is due to specific activation of the ubiquitin system, as the p27 that is found in these tumors is of the WT species.

The ubiquitin system has also been implicated in the pathogenesis of several neurodegenerative diseases. A frameshift mutation in a 'hot spot' motif in the transcript of ubiquitin and β-amyloid precursor protein (β-APP) has been described in many patients with late onset Alzheimer's disease.[81] A missense mutation in the gene encoding for the UCH-L1 has been described in a German family with Parkinson's disease.[82] Ubiquitin adducts have been described in many neurodegenerative diseases. Immunohistochemical analyses have revealed the existence of ubiquitinated neurofibrillary tangles, senile plaques, and paired helical filaments in Alzheimer's disease patients.[83] Similar analyses revealed ubiquitin-modified Huntingtin in Huntington's disease[84] and ubiquitinated Ataxin-1 in Spinocerebellar Ataxia type-1.[85] Interestingly, both Huntingtin and ataxins have an N-terminal poly-glutamine repeat that probably interferes with their normal catabolism. It is not clear whether these conjugates play a secondary pathogenetic role or whether they represent non-toxic scavenger products of the mutated abnormal/misfolded proteins that cannot be degraded by the 26S proteasome.

Microdeletions in chromosome 22q11 are the most common defects associated with cardiac and craniofacial anomalies. Two syndromes, DiGeorge syndrome (DGS) and velo-cardio-facial syndrome (VCFS) have been attributed to deletions in this region. The *UFD1L* (*ubiquitin fusion degradation*) gene is deleted in most of the patients with 22q11 deletion.[86] The UFD pathway is involved in the degradation of model proteins to which ubiquitin is stably fused at the N-terminal residue.[87] While the native cellular substrates of this pathway have not been identified, they may include proteins to which the first ubiquitin moiety is fused to the N-terminal residue.[11] UFDL1 is an enzyme that functions in a post-ubiquitination step and can be, for example, an isopeptidase.

The *CF* (*Cystic Fibrosis*) gene encodes the CFTR (CF Transmembrane Conductance Regulator) which is a chloride channel. Normally, only a small fraction of the WT protein matures to the cell surface, whereas most of the protein is degraded from the ER by the ubiquitin system.[88] The most frequent mutation in CFTR is ΔF508. Despite normal ion channel function, $CFTR^{\Delta F508}$ does not reach the cell surface, and is retained in the ER from which it is degraded. It is possible that the efficient degradation that results in complete lack of cell surface $CFTR^{\Delta F508}$, contributes to the pathogenesis of the disease.

Activation of the ubiquitin pathway is also observed during severe muscle wasting that occurs in certain forms of sepsis, cachexia, renal insufficiency, and following denervation.[67] The signals involved in eliciting the accelerated degradation of muscle proteins have not been identified.

Two interesting examples illustrate how viruses exploit the ubiquitin system to escape immune surveillance, most likely resulting in persistence and/or exacerbation of the infection. The Epstein Barr Nuclear Antigen 1 (EBNA-1) protein persists in healthy carriers for life, and is the only viral protein detected in all EBV-associated malignancies. The persistence of EBNA-1 most probably contributes to some of the virus-related pathologies. Unlike EBNAs-2-4, which are strong immunogens, EBNA-1 does not elicit a CTL response. A long C-terminal Gly-Ala repeat in EBNA-1 inhibits ubiquitin-mediated degradation and subsequent antigen presentation.[89] Interestingly, a Gly rich region in p105 prevents its degradation and serves as a 'stop' signal that is essential for its limited processing (Refs. 64, 65 and see above). A second example of how viruses utilize the ubiquitin pathways involves the human cytomegalovirus (CMV). This virus encodes two ER-resident proteins, US2 and US11 which bind to the MHC molecules in the ER and escort them to the translocation machinery. Following retrograde transport to the cytoplasm, they are ubiquitinated and degraded by the 26S′ proteasome.[90] The virus-mediated destruction of the MHC molecules prevents presentation of antigenic viral peptides at the cell surface and, thus, enables the virus to evade the immune system.

Conclusions and future perspectives

The discovery of the ubiquitin pathway with its many substrates and functions, has revolutionized our conception of intracellular protein degradation. Unlike the initial image of an unregulated, non-specific terminal scavenger process, it has become clear that proteolysis of cellular proteins is a highly complex, temporally controlled, and tightly regulated, process that plays important roles in a broad array of basic cellular processes. Despite this progress, the unknown still exceeds our knowledge of the system, and the full range of the target proteins, as well as that of the different ligases, are yet to be revealed. Only a few targeting signals have been identified, and the mechanisms that underlie the regulation of the system are still largely unknown. While the system has been implicated in the pathogenesis of several diseases, the underlying mechanisms, as well as its potential involvement in many other diseases, are still an enigma. Deciphering the complete scope of the system and its modes of action will lead not only to better understanding of basic regulatory mechanisms, but also to the development of strategies and drugs that will specifically modulate the different processes.

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89. Levitskaya J, Sharipo A, Leonchiks A, Ciechanover A, Masucci MG. Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. Proc Natl Acad Sci USA 1997;94:12616–12621.
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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Xianqiang (Jason) Li	President/CEO

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Wuhan University, Wuhan, Hubei, P.R. China	B.Sc.	1982	Virology
Wuhan Institute of Virology, Chinese Academy of Science, Wuhan, P.R. China	M.Sc.	1984	Molecular Virology
Medical College of Ohio at Toledo, Toledo, OH	Ph.D.	1990	Biochemical Pharmacology
University of California at San Francisco, San Francisco, CA	Postdoc	12/90-3/96	Molecular and Cellular Biology

A: Positions and Honors:

Professional experience

Research Associate (12/84-2/86), Wuhan Institute of Virology, China
Postdoctoral Fellow (12/90-3/96), University of California at San Francisco, CA
Research Scientist II (4/96-10/97), Clontech Laboratories, Inc.
Research Scientist III (11/97-1/99), Clontech Laboratories, Inc., Palo Alto, CA
Group Leader (1/99-9/99), Clontech Laboratories, Inc., Palo Alto, CA
R&D Director (9/99-7/00), Genemed Biotechnologies, Inc., South San Francisco, CA
President/CEO (11/00-present), Panomics Inc., Redwood City, CA

Honors and fellowships:

1996-present two US patents issued and 10 patents filed

1990 **Honors** for graduate student research forum
1992-1993 **Postdoctoral fellowship**
CRCC (Cancer Research Coordination Committee)
1994-1996 **Postdoctoral fellowship**
NIH training grant CA09043
1997-1998 **Principal Investigator**, NIH SBIR phase I (1 R43 GM56049-01)
"Ice family protease assay in apoptosis"
1997-1998 **Principal Investigator**, NIH SBIR phase I (1 R43 GM57098-01)
"In vivo detection of membrane protein/protein interaction"
1998-1999 **Principal Investigator**, NIH SBIR phase I (1 R43 GM58288-01)
"Development of GFP assay to detect NFκB activation"

2000-2001 Investigator, NIH STTR (Principal Investigator P. Coffino) 1943053156A1
"Induced selective degradation of cellular proteins"
2001-2002 **Principal Investigator,** NIH SBIR phase I (1 R43 GM64036-01)
"Technologies for identifying short-lived proteins"
2002-2003 **Principal Investigator**, NIH SBIR phase I (1 R43 GM64906-01)
"A rapid induction system for gene expression"
2003-2005 **Principal Investigator**, NIH fast-track SBIR phase I&II (1 R44 GM068159-01)
"An interaction network of transcription factors"
2004-2004 Investigator, NIH SBIR phase I (1 R43 GM070200-01)
"Development of transcription induction reporter array technology"
2004-2004 Investigator, NIH STTR phase I (1 R41 GM008996-01)
"Marketable protein array technology for profiling protein interaction"

B: Publications

1. John B. Hynes, Shirish A. Patil, Alenka Tomazic, Arvind Kumar, Alpana Pathak, Xuehai Tan, **Li Xianqiang**, Manohar Ratnam, Tavnar J. Delcamp, and James H. Freisheim (1988) Inhibition of murine thymidylate synthetase and human dehydrofolate reductase by 5'8'-dideaza analogues of folic acid and aminopterin. J. Med Chem., 31, 449-452.
2. Sham S. Kakar, Fakhari Mahdi, **Xianqiang Li**, and Keith D. Garlid (1989) Reconstitution of the mitochondrial non-selective Na+/H+ (K+/H+) antiporter into proteoliposomes. J. Biol. Chem., 264, 5846-5851.
3. **Xianqiang Li**, Mohammed G. Hegazy, Mahdi, Petr Jezek, Richard Lane, and Keith Garlid (1990) Purification of a reconstitutively active K+/H+ antiporter from rat liver mitochondria. J. Biol. Chem. 265, 15316-15322.
4. **Xianqiang Li** (1990) Purification, cloning and sequencing of mitochondrial K+/H+ antiporter. (Ph.D. dissertation).
5. **Xianqiang Li** and Philip Coffino (1992) Regulated degradation of ornithine decarboxylase requires interaction with the polyamine-inducible protein antizyme. Mol. Cell. Biol., 12, 3556-3562.
6. **Xianqiang Li** and Philip Coffino (1993) Degradation of ornithine decarboxylase: exposure of the C-terminal target by a polyamine-inducible inhibitor protein. Mol. Cell. Biol., 13, 2377-2383.
7. **Xianqiang Li** and Philip Coffino (1994) Distinct domains of antizyme for binding and proteolysis of ornithine decarboxylase. Mol. Cell. Biol., 14, 87-92.
8. Shao-bing Hua, **Xianqiang Li**, Philip Coffino, and Ching C. Wang (1995) Rat antizyme inhibits the activity but does not promote the degradation of mouse ornithine decarboxylase in Trypanosoma brucei. J. Biol. Chem., 270, 10264-10271.
9. **Xianqiang Li**, Barbara Stebbins, Laura Hoffman, Greg Pratt, Martin Rechsteiner, and Philip Coffino (1996) The N terminus of antizyme acts as a modulator in protein degradation. J. Biol. Chem., 271, 4441-4446.
10. **Xianqiang Li** and Philip Coffino (1996) Identification of a region of p53 that confers lability. J. Biol. Chem., 271, 4447-4451.
11. **Xianqiang Li** and Philip Coffino (1996) High-risk human papilloma virus E6 protein has two binding sites within p53, of which only one determines degradation. J. Virol., 70, 4509-4516.

12. **Xianqiang Li**, Guohong Zhang, Nhatanh Ngo, Xiaoning Zhao, Steven R. Kain, Chiao-chain Huang (1997) Deletions of the Aequorea victoria green fluorescent protein define the minimal domain required for fluorescence. J. Biol. Chem. 272, 28545-28549.
13. **Xianqiang Li**, Nhatanh Ngo, Chrestie Hou, Sara Cunningham, Ruo Bu Zhang, Zhu Li, and Chiao-chain Huang (1997) Screening for positive transfected clones with co-expressed green fluorescent protein. BioTechniques, 24, 52-55.
14. **Xianqiang Li**, Xiaoning Zhao, Yu Fang, Xin Jiang, Tommy Duong, Chiao-Chain Huang, Steven R. Kain. (1998) Generation of destabilized EGFP as a transcription reporter. J. Biol. Chem. 273, 34970-34975.
15. Xiaoning Zhao, Tommy Duong, Chiao-Chian Huang, Steven R. Kain, **Xianqiang Li**. (1999) Comparison of EGFP and Its Destabilized Form as Transcription Reporters. Methods Enzymol. 302, 32-38.
16. Xiaoning Zhao, Xin Jiang, Chiao-Chian Huang, Steven R. Kain, **Xianqiang Li**. (1999) Generation of A Destabilized Form of EGFP. Methods Enzymol. 302, 438-444.
17. Yu Fang, Chiao-Chain Huang, Steve, R. Kain, and **Xianqiang Li**. (1999) Use of co-expressed enhanced green fluorescent protein as a marker for identifying transfected cells. Methods Enzymol. 302, 207-212.
18. **Xianqiang Li**, Yu Fang, Xiaoning Zhao, Xin Jiang, Tommy Duong, Steven R. Kain. (1999) Characterization of NFkB activation by detection of green fluorescent protein tagged IkB degradation in living cells. J. Biol. Chem. 274, 21244-21250.
19. **Xianqiang Li** (2001) New Technology for multiplexed functional analysis of proteins (review). Molecular Cardiovascular Medicine, 2, 23-28.
20. Robert Lam and **Xianqiang Li**, (2002) An array-based method for specifically profiling multiple transcription factor activity. American Biotechnology Laboratory, 20 (8): 22-26.
21. Xin Jiang, Michael Norman, **Xianqiang Li**. (2003) Use of an array technology for profiling and comparing transcription factors activated by TNFalpha and PMA. Biochim Biophys Acta. 1642(1-2): 1-8.
22. Chamnongpol, S., and **Xianqiang Li**. (2004) SH3 Domain Protein-Binding Arrays. Methods in Molecular Biology, 278, 183-90.
23. Xin Jiang, Michael Normal, Leslie Roth, **Xianqiang Li**. Protein/DNA Array Based Identification of Transcription Factor Activities Regulated by Interaction with the Glucocorticoid Receptor. The Journal of Biological Chemistry. Web published. (in press).
24. Xin Jiang, Philip Coffino and **Xianqiang Li.** Development of a GFP-Based Method for Screening Short-Lived Proteins. Genome Biology. (in press).